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<b>(54) Title:</b> CLONING AND REGULATION OF AN ENDOTHELIAL CELL PROTEIN C/ACTIVATED PROTEIN C RECEPTOR			
<b>(57) Abstract</b> <p>Human protein C and activated protein C were shown to bind to endothelium specifically, selectively and saturably (K<sub>d</sub>=30 nM, 7000 sites per cell) in a Ca<sup>2+</sup> dependent fashion. Expression cloning revealed a 1.3 kb CDNA that coded for a novel type I transmembrane glycoprotein capable of binding protein C. This protein appears to be a member of the CD1/MHC superfamily. Like thrombomodulin, the receptor involved in protein C activation, the endothelial cell protein C receptor (EPCR) function and message are both down regulated by exposure of endothelium to TNF. Identification of EPCR as a member of the CD1/MHC superfamily provides insights into the role of protein C in regulating the inflammatory response, and determination of methods for pharmaceutical use in manipulating the inflammatory response.</p>			

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CLONING AND REGULATION OF AN ENDOTHELIAL CELL  
PROTEIN C/ACTIVATED PROTEIN C RECEPTOR

Background of the Invention

The present invention is generally in the area of cloning, expression, and regulation of an endothelial cell protein C/activated protein C  
5 receptor.

Protein C plays a major role in the regulation of blood coagulation. Patients deficient in protein C usually exhibit life threatening thrombotic-complications in infancy  
10 (Seligsohn et al., (1984) N. Engl. J. Med. 310, 559-562; Esmon, (1992) Trends Cardiovasc. Med. 2, 214-220) that are corrected by protein C administration (Dreyfus et al., (1991) N. Engl. J. Med. 325, 1565-1568). In addition, activated  
15 protein C (APC) can prevent the lethal effects of *E. coli* in baboon models of gram negative sepsis (Taylor et al., (1987) J. Clin. Invest. 79; U.S. Patent No. 5,009,889 to Taylor and Esmon) and preliminary clinical results suggest that protein C  
20 is effective in treating certain forms of human septic shock (Gerson et al., (1993) Pediatrics 91, 418-422). These results suggest that protein C may both control coagulation and influence inflammation. Indeed, inhibition of protein S, an  
25 important component of the protein C pathway, exacerbates the response of primates to sublethal levels of *E. coli* and augments the appearance of TNF in the circulation (Taylor et al., (1991) Blood 78, 357-363). The mechanisms involved in  
30 controlling the inflammatory response remain unknown.

Protein C is activated when thrombin, the terminal enzyme of the coagulation system, binds to an endothelial cell surface protein, thrombomodulin  
35 (Esmon, (1989) J. Biol. Chem. 264, 4743-4746; Dittman and Majerus, (1990) Blood 75, 329-336;

Dittman, (1991) Trends Cardiovasc. Med. 1, 331-336). In cell culture, thrombomodulin transcription is blocked by exposure of endothelial cells to tumor necrosis factor (TNF) (Conway and Rosenberg, (1988) Mol. Cell. Biol. 8, 5588-5592) and thrombomodulin activity and antigen are subsequently internalized and degraded (Lentz et al., (1991) Blood 77, 543-550, Moore, K.L., et.al., (1989) Blood 73, 159-165). In addition, C4bBP, a regulatory protein of the complement system, binds protein S to form a complex that is functionally inactive in supporting APC anticoagulant activity *in vitro* (Dahlbäck, (1986) J. Biol. Chem. 261, 12022-12027) and *in vivo* (Taylor, et al., 1991). Furthermore, C4bBP behaves as an acute phase reactant (Dahlbäck, (1991) Thromb. Haemostas. 66, 49-61). Thus, proteins of this pathway not only appear to regulate inflammation, but they also interact with components that regulate inflammation, and they themselves are subject to down regulation by inflammatory mediators.

Given the central role of the protein C pathway in regulating the host response to inflammation and the critical role of the pathway in controlling blood coagulation, it is important to identify and characterize all of the components that interact with the system. This is especially true since the molecular basis of the anti-inflammatory effects of the protein C pathway components have yet to be elucidated at the molecular level.

It is therefore an object of the present invention to provide a cellular receptor for protein C and activated protein C.

It is a further object of the present invention to provide nucleotide sequences encoding

the cellular receptor and amino acid characterization of the receptor which allows expression of recombinant native and modified forms of the receptor.

5 It is another object of the present invention to provide methods of modulating the inflammatory response involving protein C and activated protein C.

#### Summary of the Invention

10 An endothelial cell protein C binding protein (referred to herein as "EPCR") has been cloned and characterized. The protein is predicted to consist of 238 amino acids, which includes a 15 amino acid signal sequence at the N-terminus, and a  
15 23 amino acid transmembrane region which characterizes the receptor as a type 1 transmembrane protein. The protein binds with high affinity to both protein C and activated protein C (Kd=30 nM) and is calcium dependent. The message  
20 and binding function of the receptor are both down regulated by cytokines such as TNF.

These results identify a new member of a complex pathway that, like other members of the pathway, is subject to regulation by inflammatory  
25 cytokines, and can therefore be used to modulate inflammatory reactions in which protein C or activated protein C is involved. Inhibition of the inflammatory response can be obtained by infusing soluble EPCR. Alternatively, localizing  
30 EPCR to surfaces in contact with blood will render the surfaces anticoagulant by virtue of the ability of EPCR to bind and concentrate the anticoagulant activated protein C at the surface. Alternatively, the function of EPCR can be enhanced by  
35 overexpressing the EPCR in endothelium that could

be used to coat vascular grafts in patients with vascular disease or on stents in cardiac patients.

#### Brief Description of the Drawings

Figures 1A, 1B and 1C are flow cytometric analyses of F1-APC (fluorescent labelled activated protein C) binding to HUVEC (human umbilical vein endothelial cells). Figure 1A is a graph of cell number versus log of fluorescence intensity, demonstrating F1-APC binding to HUVEC. HUVEC ( $1 \times 10^5$ ) were incubated at room temperature without (dotted line) or with 160 nM of F1-APC (solid line) in the presence of 1.3 mM  $\text{CaCl}_2$ . After washing, bound APC was analyzed by flow cytometry. Figure 1B is a graph of fluorescence intensity versus F1-APC concentration (nM) demonstrating the concentration dependence of F1-APC binding to HUVEC. HUVEC were incubated with F1-APC in the absence (open circles) or presence of 1.3 mM  $\text{CaCl}_2$  (closed circles) and binding was measured as in A. Mean channel fluorescence intensity is plotted for each F1-APC concentration (between 0 and 800 nM). Figure 1C is a graph of the percent of mean fluorescence versus unlabeled protein concentration ( $\mu\text{g/ml}$ ), demonstrating the effects of unlabeled proteins on F1-APC binding to HUVEC. F1-APC binding to HUVEC was carried out in the presence of the indicated concentrations (between 0 and 100  $\mu\text{g/ml}$ ) of unlabeled APC, protein C, protein S, factor X and Xa or recombinant Gla-domainless protein C (rGDPC).

Figures 2A, 2B, 2C and 2D are graphs of  $^{125}\text{I}$ -APC Binding to HUVEC Monolayers. Figure 2A is a graph of the bound APC ( $\text{cpm} \times 10^{-3}$ ) versus time (min), showing the time course of  $^{125}\text{I}$ -APC binding to HUVEC. HUVEC monolayers ( $1.2 \times 10^5$  cells) were incubated at  $4^\circ\text{C}$  with 32 nM (filled squares) or 8

nM (open squares)  $^{125}\text{I}$ -APC. At the indicated times, cells were washed and bound radioactivity was measured. Figure 2B is a graph of bound APC (cpm  $\times 10^{-3}$ ) versus unlabeled protein (nM) demonstrating the effects of unlabeled APC and rGDPC on  $^{125}\text{I}$ -APC binding to HUVEC. HUVEC were incubated at  $4^\circ\text{C}$  for one hour with  $^{125}\text{I}$ -labeled APC in the presence of the indicated concentrations (between 01 and approximately 1000 nM) of unlabeled APC (open circles) or rGDPC (closed circles). After washing, bound radioactivity was measured. Figure 2C is a graph of bound APC (fmol/well) versus free APC (nM) demonstrating the concentration dependence of  $^{125}\text{I}$ -APC binding to HUVEC. Monolayers of HUVEC were incubated with the concentrations of  $^{125}\text{I}$ -APC indicated as described above. Specific binding was determined as described below. Figure 2D is a Scatchard analysis of  $^{125}\text{I}$ -APC binding to HUVEC. Each value was calculated from the data shown in Figure 2C.

Figures 3A and 3B are flow cytometric analyses of F1-APC binding to 293T cells transfected with a cDNA clone of EPCR. Cells were transfected with a clone EPCR/pEF-BOS or pEF-BOS (negative control) by the calcium/phosphate method. After 24 h, cells were harvested and F1-APC binding was performed in the absence (dotted lines) or presence of 1.3 mM  $\text{CaCl}_2$  (solid lines).

Figures 4a-4c are the predicted protein structure of EPCR based on nucleotide sequence (SEQ ID NO. 1), predicted amino acid sequence (SEQ ID NO. 2) and a hydropathy plot of EPCR. The signal sequence and transmembrane region are indicated with the solid bars.

Figures 5a-5b are a comparison of the amino acid sequence of EPCR to the amino acid sequences of other members of the CD1 family and CCD41. The EPCR sequence is shown in the first line and

compared to murine CCD41 (second line), human CD1d (third line) and murine CD1.2 (fourth line).

Identities with EPCR are indicated by open boxes.

Residues that are conserved between EPCR and all of the human CD1 family members are indicated by a double asterisk. Residues shared with one or more members of the CD1 family are indicated by a single asterisk.

Figure 6 is a comparison of the amino acid sequence of human EPCR (first line) to the amino acid sequence of murine EPCR (second line). Identities are indicated by lines. Similarities are indicated with dots.

#### Detailed Description of the Invention

##### I. Cloning and Characterization of EPCR.

Human protein C and activated protein C are shown to bind to endothelium specifically, selectively and saturably ( $K_d = 30$  nM, 7000 sites per cell) in a  $Ca^{2+}$  dependent fashion. FL-APC binding to various human cell lines were examined, and found that the binding was HUVEC specific. A human kidney cell line transformed with SV40 large T antigen, 293T cells, expressed very few of these binding sites. A HUVEC cDNA library was constructed using the powerful mammalian expression vector, pEF-BOS (Mizushima and Nagata, (1990) Nucleic Acids Res. 18, 5322). Plasmid DNA was prepared from subpools of independent colonies (2,500 colonies per pool), and transfected into 293T cells, using the method of Kaisho et al., (1994) Proc. Natl. Acad. Sci. (USA) 91, 5325. FL-APC binding was analyzed on a flow cytometer. One of eight subpools gave a positive signal. This subpool was divided into 20 subpools and rescreened. After three rounds of enrichment, one positive clone, EPCR-1, was isolated. EPCR-1



carries a 1.3 kb insert. When transfected into 293T cells, this clone was capable of expressing the calcium-dependent binding site for FL-APC on the 293T cell surface.

5 Expression cloning revealed a 1.3 kb cDNA that coded for a type I transmembrane glycoprotein capable of binding protein C. This protein appears to be a member of the CD1/MHC superfamily. Like thrombomodulin, the receptor involved in protein C  
10 activation, the endothelial cell protein C receptor (EPCR) function and message are both down regulated by exposure of endothelium to TNF. Identification of EPCR as a member of the CD1/MHC superfamily provides insights into the role of this receptor  
15 for protein C in regulating the inflammatory response.

#### Materials and Methods

##### Protein Preparation

Human protein C (Esmon et al., (1993)  
20 Meths. Enzymol. 222, 359-385), APC (Esmon et al., 1993), recombinant gla domainless protein C (rGDPC) (Rezaie et al., (1992) J. Biol. Chem. 267, 11701-11704), protein S (Taylor et al., 1991), factor X and factor Xa (Le Bonniec et al., (1992)  
25 J. Biol. Chem. 267, 6970-6976) were prepared as described in the cited publications.

Selective labeling of the active site of APC with fluorescein was performed by the method of Bock (Bock, P.E. (1988) Biochemistry 27, 6633-6639). In brief, N<sup>o</sup>-  
30 [(acetylthio)acetyl]-D-Phe-Pro-Arg-CH<sub>2</sub>Cl (200 μM) was reacted with 40 μM APC for 1 hour at room temperature. After dialysis, the covalently modified APC was incubated at room temperature for  
35 one hour with 200 μM 5-(iodoacetamido)fluorescein (Molecular Probes). Free fluorescein was removed by gel filtration on a PD-10 column (Pharmacia).

With this method, each molecule of fluoresceinated APC (FI-APC) contains a single dye at the active site and hence all of the fluorescent molecules behave identically.

5 Iodogen (Pierce) was used to radiolabel APC with Na[<sup>125</sup>I] (Amersham) according to the manufacture's protocol in the presence of 5 mM CaCl<sub>2</sub>. Free <sup>125</sup>I was removed by gel filtration on a PD-10 column. The specific activity of the <sup>125</sup>I-APC  
10 was 1 x 10<sup>4</sup> cpm/ng protein.

#### Cell Culture

Human umbilical vein endothelial cells (HUVEC) were isolated from fresh umbilical-cords by collagenase treatment and cultured in medium 199  
15 containing 15% fetal bovine serum, 10 µg/ml heparin, and 0.5% endothelial cell growth supplement prepared from bovine brain extract (Maciag et al., (1979) Proc. Natl. Acad. Sci. (USA) 76, 5674-5678). HOS (ATCC CRL 1543), HEP-2 (ATCC  
20 CCL 23) and 293 cells (ATCC CRL 1573) transformed with SV40 large T antigen (293T, a gift from Dr. Kenji Oritani) were maintained in Earl's MEM supplemented with 10% fetal bovine serum. The human lymphocyte cell lines, Jurkat, MOLT3 (ATCC  
25 CRL 1552), Jijoye (ATCC CCL 87), Raji (ATCC CCL 86), U-937 (ATCC CRL 1593), HL-60 (ATCC CCL 240), and HEL (ATCC TIB 180), were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum.

#### 30 Flow Cytometric Analysis of FI-APC Binding to Cells

Adherent cells were harvested by incubation at 37°C for 5 min in phosphate buffered saline (PBS) containing 0.02% EDTA. Cells were  
35 washed twice with EDTA/PBS and then once with Hank's balanced salt solution (HBSS). They were resuspended in HBSS containing 1% bovine serum albumin (BSA) and 0.02% sodium azide (binding

buffer). Cells ( $1 \times 10^5$ ) were incubated at room temperature for 45 min with Fl-APC in the dark. After washing, they were resuspended in the binding buffer containing  $0.5 \mu\text{g/ml}$  of propidium iodide.

- 5 Bound Fl-APC was analyzed on a flow cytometer, FACScan (Becton Dickinson). Living cells were gated on a dot plot display of forward-scatter (FSC) versus fluorescence-2 (FL2), and Fl-APC binding was detected on the fluorescence-1 (FL-1)
- 10 channel. All experiments were performed in duplicate.

#### **$^{125}\text{I}$ -APC binding to HUVEC**

- Monolayers of HUVEC in 24-well microplates (Costar) ( $1 \times 10^5$  cells per well) were
- 15 washed twice with EDTA/PBS and once with ice-cold HBSS. Cells were then incubated at  $4^\circ\text{C}$  for one hour in the binding buffer with  $^{125}\text{I}$ -APC. After washing three times with ice-cold HBSS, cells were released with the EDTA buffer, and the bound
- 20 radioactivity was measured in a gamma counter (Isodata 500). To determine non-specific, calcium-independent adsorption of radioactivity, the cells were washed with EDTA/PBS and residual radioactivity in the cell pellet was measured.
- 25 Non-specific binding of radioactivity was consistently less than 5% of the specific binding. The data was analyzed using the Enzfitter program (Elsevier Biosoft, Cambridge, U.K.).

#### **Construction of HUVEC CDNA Library**

- 30 Poly-A RNA was isolated from HUVEC ( $1 \times 10^8$  cells) using the FastTrack<sup>TM</sup> mRNA isolation kit (Invitrogen). cDNA was synthesized from  $3 \mu\text{g}$  of poly-A RNA using a Librarian<sup>TM</sup> I kit (Invitrogen). A BstX I adaptor was ligated, double stranded cDNA
- 35 was fractionated by agarose gel electrophoresis, and cDNA longer than 700 bp was ligated into a mammalian expression vector, pEF-BOS (Mizushima and

Nagata, 1990; this vector was a kind gift from Dr. S. Nagata). The construct was transfected into *E. coli* DH10B by electroporation (Bio-Rad Gene Pulser™). The library- consisted of  $8 \times 10^6$   
5 independent colonies with an average size of 2.0 kb.

#### Expression Cloning and Sequence Analysis

Approximately  $2 \times 10^4$  independent colonies were divided into eight subpools (each containing  
10 2,500 independent colonies) and plasmid DNA was prepared from each subpool. Sub-confluent 293T cells in 24-well microplates were transfected with 1  $\mu$ g of the DNA by the calcium/phosphate method (Graham and Van Der Eb, (1973) *Virology* 52, 456-  
15 467). After 20 hours, the medium was changed, and culture was continued for another 24 hours. The subpools were screened for F1-APC binding by FACS analysis as described above. The positive library pool was then divided into 20 new pools and  
20 rescreened. After three rounds of screening, 96 individual clones were tested and one positive clone was identified.

The insert (1.3 kb) was subcloned into pBluescript™ (Stratagene), and the nucleotide  
25 sequence was determined using a Sequenase™ version 2.0 DNA Sequencing kit (USB). Nucleotide and protein database search employed the BLAST™ (NCBI) and FASTA™ programs (GCG) with GenBank, EMBL, and SwissProt databases.

#### 30 Northern Blot Analysis

Total RNAs (15  $\mu$ g) from various cells were isolated, electrophoresed through formaldehyde agarose gels and transferred to a nylon membrane (Hybond-N™, Amersham). The 483 bp *Xba* I fragment  
35 from the 5' end of the EPCR cDNA was labeled by random priming according to the manufacturer's

instructions (Multiprime™ DNA labeling system, Amersham) and used for hybridization.

#### Protein C and APC Binding to HUVEC

Endothelial cells in suspension bound

5 FL-APC, as monitored by flow cytometry, and demonstrated in Figure 1A. Binding was saturable and  $\text{Ca}^{2+}$  dependent, as shown by Figure 1B. Optimal binding required at least 1 mM  $\text{Ca}^{2+}$ . FL-APC was displaced from the cell surface by APC and protein  
10 C equivalently, as shown by Figure 1C. The homologous Gla-domain containing proteins, protein S, factor X, and its active form, factor Xa, failed to displace bound F1-APC, suggesting that there is a specific binding site for APC on the endothelial  
15 cell surface. Protein C binding was dependent on the Gla domain, since recombinant gla-domainless protein C (rGDPC) failed to displace F1-APC.

Detailed binding studies were also performed with  $^{125}\text{I}$ -labeled APC and monolayers of  
20 HUVEC, as shown by Figures 2A, 2B, 2C and 2D. The binding analysis indicated 7,000 sites per cell and a  $K_d=30$  nM. This affinity is similar to that estimated from Figure 1.

Endothelial cell surface thrombomodulin  
25 can interact with protein C and APC. The  $K_d$  (greater than 1  $\mu\text{M}$ ) (Hogg et al., (1992) J. Biol. Chem. 267, 703-706; Olsen et al., (1992) Biochemistry 31, 746-754), however, is much higher than that of the binding site described above with  
30 respect to the new receptor. Furthermore, polyclonal and monoclonal antibodies against thrombomodulin that inhibit protein C activation did not inhibit the binding. Protein S also can interact with protein C and APC (Dahlbäck et al.,  
35 (1992) Biochemistry 31, 12769-12777), but F1-APC binding to HUVEC was not influenced by protein S addition. Furthermore, polyclonal and monoclonal

antibodies to protein S did not inhibit the binding. These results indicate the binding site for protein C and APC on endothelium is distinct from these known molecules.

5                   **Nucleotide and Predicted Protein  
Structure Analysis of EPCR**

The insert was subcloned into pBluescript, and the nucleotide sequence was determined, as shown in Sequence ID No. 1. The  
10 cDNA shown in Sequence ID No. 1 consists of 1302 bp, including a translation initiation ATG codon (AGGATGT, (Kozak, (1986) Cell 44, 283-292) at the 5'-end at nucleotides 25-27 of Sequence ID No. 1. A potential polyadenylation signal sequence,  
15 AATAAA, (Proudfoot and Brownlee, (1976) Nature 263, 211-214) begins at nucleotide 1267 of Sequence ID No. 1, just 18 bp upstream of the poly(A) sequence.

The cDNA is predicted to code for a protein of 238 amino acids (Sequence ID No. 2),  
20 which includes a 15 amino acid signal sequence (von Heijne, (1986) Nucleic Acids Res. 14, 4683-4690) at the N-terminal. Therefore, the mature protein is predicted to contain 223 amino acids. Sequence ID No. 2 is the predicted amino acid sequence of EPCR.  
25 Amino acids 1-15 of Sequence ID No. 2 (MLTTLLPILLSGWA) are the putative signal sequence determined by the method of von Heijne (von Heijne, 1986). Amino acids 211-236 of Sequence ID No. 2 (LVLGVLVGGFIIAGVAVGIFLCTGGR) are the putative  
30 transmembrane domain. Potential N-glycosylation sites are present at amino acids 47-49, 64-66, 136-138, and 172-174 of Sequence ID No. 2. Extracellular cysteine residues are present at amino acids 17, 114, 118, and 186 of Sequence ID  
35 No. 2. A potential transmembrane region (Engelman et al., (1986) Annu. Rev. Biophys. Chem. 15, 321-53 ) consisting of 23 amino acids was identified at

the C-terminal end (beginning at amino acid 216 of Sequence ID No. 2).

The protein is predicted to be a type 1 transmembrane protein. The extracellular domain  
5 contains four potential N-glycosylation sites and four Cys residues. The cytoplasmic region contains only three amino acids and terminates with a Cys, which could be acylated to something or involved in heterodimer formation with another peptide.

10 Although described with reference to cloning and expression of the protein encoding sequence, larger amounts of protein can be obtained by expression in suitable recombinant host systems, such as mammalian, yeast, bacteria, or insect  
15 cells. Isolation can be facilitated by making antibodies to the recombinant protein which are then immobilized on substrates for use in purification of additional receptors, as described below.

20 As used herein, the nucleotide sequences encoding the receptor include the sequence shown in Sequence ID No. 1, and sequences having conservative substitutions, additions or deletions thereof which hybridize to Sequence ID No. 1 under  
25 stringent conditions. As used herein, the amino acids sequences constituting the receptor include the sequence shown in Sequence ID No. 2, and sequences having conservative substitutions, additions or deletions thereof which form a  
30 receptor having functionally equivalent biological activity. It is well known to those skilled in the art what constitutes conservative substitutions, additions or deletions, and which could be readily ascertained as encoding, or forming, a functionally  
35 equivalent receptor molecule using the functional assays described herein.

The hydropathic plot shown in Figures 4a-4c was performed according to the method of Goldman et al (Engelman et al., 1994) (solid line) and that of Kyte and Doolittle (1982) J. Mol. Biol. 157, 105-132 (dotted line).

DNA and protein database searches revealed that the sequence is related to the centrosome-associated, cell cycle dependent murine protein, CCD41, also referred as centrocyclin (Rothbarth et al., (1993) J. Cell Sci. 104, 19-30), as shown by Figures 5a-5b. The similarity in the published sequence of murine CCD41 with human EPCR led to the cloning and sequencing of the murine EPCR. The sequence of murine EPCR is shown in Figure 6. It is distinct from the published sequence of CCD41.

The EPCR amino acid sequence was also related to, but quite distinct from, the CD1/MHC superfamily and the murine CD1.2, as also shown by Figures 5a-5b. Based on the homology to CD1/MHC, it is likely that EPCR contains two domains consisting of residues 17-114 and 118-188. Of the CD1 family members, CD1d is the most similar to EPCR. In the mouse, CCD41 is associated exclusively with the centrosome during G<sub>1</sub> but becomes detectable elsewhere during the cell cycle, reaching a maximum during G<sub>2</sub>, except during the G<sub>2</sub>/M phase (Rothbarth et al., 1993). EPCR expression appears restricted to endothelium, which would not be expected for a cell cycle associated protein.

The identification of the protein C receptor on endothelium suggests that the endothelial cell binds protein C/APC through three distinct mechanisms. In addition to EPCR, protein S can bind APC/protein C on negatively charged membrane surfaces that include the endothelium (Stern et al., (1986) J. Biol. Chem. 261, 713-718),



but this is not cell type specific (Dahlbäck et al., 1992). Thrombomodulin in complex with thrombin can bind protein C and APC (Hogg et al., 1992). On endothelium, the protein S binding sites  
5 (Nawroth and Stern, (1986) J. Exp. Med. 163, 740-745), thrombomodulin (Esmon, 1989) and EPCR are all down regulated by cytokines, indicating that inflammation can impair protein C pathway function at multiple levels.

10           The homology to the CD1/MHC family of proteins is especially interesting since it provides indications as to the function of EPCR. The CD1/MHC family has three extracellular domains termed  $\alpha 1, 2$  and 3. The extracellular domain of EPCR  
15 contains four Cys residues that appear to correspond to two distinct domains. EPCR lacks the third domain of the CD1/MHC family, but the two domains have significant homology to the  $\alpha 1$  and  $\alpha 2$  domains of the CD1 protein family and the  $\alpha 2$  domain  
20 of the MHC class 1 protein, suggesting that these proteins evolved from a common ancestor. The first domain of EPCR, residues 17-114, contains two potential N glycosylation sites and is rich in  $\beta$  strand structure, suggesting that it may form a  $\beta$   
25 sheet. Despite the  $\beta$  strand structure, consensus sequences (Williams and Barclay, (1988) Ann. Rev. Immunol. 6, 381-405) for the immunoglobulin superfamily of receptors are absent. The second domain of EPCR, residues 118-188, contains two  
30 additional N glycosylation sites and, like the CD1/MHC family, this domain is predicted to have limited  $\beta$  structure.

## II. Modulation of Inflammation using EPCR.

35           In vitro studies have suggested anti-inflammatory activities for APC. For instance, an unusual carbohydrate sequence on protein C can inhibit inflammatory cell adhesion to

selectins (Grinnell et al., (1994) Glycobiology, 4, 221-226) Modest inhibitory effects of APC have been reported on TNF production (Grey et al., (1993) Transplant. Proc. 25, 2913-2914). EPCR could contribute to these anti-inflammatory mechanisms. Since the homologous protein family, CD1, can be linked to CD8 (Ledbetter et al., (1985) J. Immunol. 134, 4250-4254), it is also possible that the protein C receptor is linked to another protein and signal through this second protein. One of the CD1 family members, CD1d, has been reported to promote T cell responses, possibly involving binding to CD8 (Panja et al., (1993) J. Exp. Med. 178, 1115-1119). CD1b has recently been reported to serve as an antigen presenting molecule (Porcelli et al., (1992) Nature 360, 593-597). The ability to bind protein C/APC could then be linked either directly or indirectly to signalling via direct interaction with cells of the immune system. Since the MHC class of proteins is involved in presentation of proteins to cell receptors, the concept of presentation of protein C/APC to inflammatory cells as a means of elaborating anti-inflammatory activity may also be involved. This includes modulation of enzyme specificity such as occurs with thrombin-thrombomodulin interaction (Esmon, 1989). In this case, the EPCR-APC complex might cleave biologically active peptides from unknown substrates.

#### 30 EPCR mRNA Levels and APC Binding

To determine the cellular specificity of EPCR expression, the intensity of FL-APC binding to HUVEC was compared to several human cell lines. Fl-APC bound strongly only to HUVEC, and not to any of the T, B, or monocytic cell lines tested. Cells were incubated at room temperature without or with 160 nM Fl-APC in the presence of 1.3 mM  $\text{CaCl}_2$ .

Binding was analyzed by flow cytometry. Slight binding was demonstrated with the osteosarcoma line, HOS and the epidermoid carcinoma cell line, HEP-2.

5           Total RNA was extracted from these cells and hybridized with the EPCR cDNA probe for Northern Blot Analysis. EPCR mRNA was detected by Northern blot analysis for HUVEC, Jurkat, HEP-2, Raji, HOS, and U937. Among the cells lines tested,  
10 EPCR mRNA was detected at high levels only in HUVEC. The calculated mRNA size of 1.3 kb was identical to the size of the isolated cDNA. After prolonged exposure, a weak signal was also detected with the osteosarcoma cell line HOS and monocyte  
15 cell line U937. Thus, both APC binding and EPCR mRNA expression are very specific for endothelium.

#### Effects of TNF on APC Binding and EPCR mRNA Levels

Several other members of the protein C  
20 anticoagulant pathway are subject to regulation by inflammatory cytokines (Esmon, 1989). For instance, endothelial cell surface thrombomodulin expression and message are known to be reduced by exposure of the cells to TNF (Conway and Rosenberg,  
25 1988; Lentz et al., 1991). To determine if a similar process occurs with EPCR, HUVEC were treated with TNF and APC binding and expression of EPCR mRNA were examined. APC binding to HUVEC decreased in a time dependent fashion. EPCR  
30 activity decreased more rapidly than thrombomodulin antigen. HUVEC were cultured for 0, 6, 24 and 48 hr, in the presence of TNF- $\alpha$  (10 ng/ml). Cells were harvested and residual F1-APC binding or thrombomodulin (TM) expression was analyzed by flow  
35 cytometry. Cell surface TM was stained with an anti-TM murine monoclonal antibody and FITC--conjugated anti-mouse IgG. The negative control is without added fluorescent ligand.

HUVEC were treated with 10 ng/ml of TNF- $\alpha$  for 0, 0.5, 1, 2, 3, 6, 10 and 24 hr, and message was extracted and detected as described above. The results demonstrated that the concentration of EPCR mRNA was also reduced by TNF treatment. Message levels and APC binding activity decreased in parallel. Therefore, the TNF mediated down-regulation of APC binding to endothelium probably occurs at the level of mRNA expression.

Enhancement of inflammatory responses by blocking binding of endogenous molecules to ECPCR can be achieved by administration of compounds binding to the receptor to a subject in need of inhibition. The degree of binding is routinely determined using assays such as those described above. Compounds which are effective include antibodies to the protein, fragments of antibodies retaining the binding regions, and peptide fragments of APC which include the Gla region.

Inhibition of the inflammatory response could be obtained by infusing soluble EPCR. Alternatively, localizing EPCR to surfaces in contact with blood would render the surfaces anticoagulant by virtue of the ability of EPCR to bind and concentrate the anticoagulant APC at the surface. Alternatively, the function of EPCR could be enhanced by overexpressing the EPCR in endothelium used to coat vascular grafts in patients with vascular disease or on stents in cardiac patients.

The DNA sequence can also be used for screening for other homologous or structurally similar receptor proteins using hybridization probes.

These methods and reagents and pharmaceuticals are more readily understood by reference to the following.

Screening of patient samples for  
expression of receptor proteins.

Patients with thrombosis or hyperinflammatory conditions could be screened for defects in the EPCR gene. Sequence ID No. 1, and consecutive portions thereof of at least about seven nucleotides, more preferably fourteen to seventeen nucleotides, most preferably about twenty nucleotides, are useful in this screening using hybridization assays of patient samples, including blood and tissues. Screening can also be accomplished using antibodies, typically labelled with a fluorescent, radiolabelled, or enzymatic label, or by isolation of target cells and screening for binding activity, as described in the examples above. Typically, one would screen for expression on either a qualitative or quantitative basis, and for expression of functional receptor. Labelling can be with  $^{32}\text{P}$ ,  $^{35}\text{S}$ , fluorescein, biotin, or other labels routinely used with methods known to those skilled in the art for labelling of proteins and/or nucleic acid sequences.

*Hybridization Probes*

Reaction conditions for hybridization of an oligonucleotide probe or primer to a nucleic acid sequence vary from oligonucleotide to oligonucleotide, depending on factors such as oligonucleotide length, the number of G and C nucleotides, and the composition of the buffer utilized in the hybridization reaction. Moderately stringent hybridization conditions are generally understood by those skilled in the art as conditions approximately 25°C below the melting temperature of a perfectly base-paired double-stranded DNA. Higher specificity is generally achieved by employing incubation conditions having higher temperatures, in other words, more stringent conditions. In general, the longer the sequence

or higher the G and C content, the higher the temperature and/or salt concentration required. Chapter 11 of the well-known laboratory manual of Sambrook et al., MOLECULAR CLONING: A LABORATORY MANUAL, second edition, Cold Spring Harbor Laboratory Press, New York (1990) (which is incorporated by reference herein), describes hybridization conditions for oligonucleotide probes and primers in great detail, including a description of the factors involved and the level of stringency necessary to guarantee hybridization with specificity.

The preferred size of a hybridization probe is from 10 nucleotides to 100,000 nucleotides in length. Below 10 nucleotides, hybridized systems are not stable and will begin to denature above 20°C. Above 100,000 nucleotides, one finds that hybridization (renaturation) becomes a much slower and incomplete process, as described in greater detail in the text MOLECULAR GENETICS, Stent, G.S. and R. Calender, pp. 213-219 (1971). Ideally, the probe should be from 20 to 10,000 nucleotides. Smaller nucleotide sequences (20-100) lend themselves to production by automated organic synthetic techniques. Sequences from 100-10,000 nucleotides can be obtained from appropriate restriction endonuclease treatments. The labeling of the smaller probes with the relatively bulky chemiluminescent moieties may in some cases interfere with the hybridization process.

*Generation of Antibodies for  
Diagnostic or Therapeutic Use*

Antibodies to the receptor proteins can also be generated which are useful in detection, characterization or isolation of receptor proteins, as well as for modifying receptor protein activity, in most cases, through inhibition of binding. Antibodies are generated by standard techniques,

using human or animal receptor proteins. Since the proteins exhibit high evolutionary conservation, it may be advantageous to generate antibodies to a protein of a different species of origin than the species in which the antibodies are to be tested or utilized, looking for those antibodies which are immunoreactive with the most evolutionarily conserved regions. Antibodies are typically generated by immunization of an animal using an adjuvant such as Freund's adjuvant in combination with an immunogenic amount of the protein administered over a period of weeks in two to three week intervals, then isolated from the serum, or used to make hybridomas which express the antibodies in culture. Because the methods for immunizing animals yield antibody which is not of human origin, the antibodies could elicit an adverse effect if administered to humans. Methods for "humanizing" antibodies, or generating less immunogenic fragments of non-human antibodies, are well known. A humanized antibody is one in which only the antigen-recognized sites, or complementarily-determining hypervariable regions (CDRs) are of non-human origin, whereas all framework regions (FR) of variable domains are products of human genes. These "humanized" antibodies present a lesser xenographic rejection stimulus when introduced to a human recipient.

To accomplish humanization of a selected mouse monoclonal antibody, the CDR grafting method described by Daugherty, et al., (1991) Nucl. Acids Res., 19:2471-2476, incorporated herein by reference, may be used. Briefly, the variable region DNA of a selected animal recombinant anti-idiotypic ScFv is sequenced by the method of Clackson, T., et al., (1991) Nature, 352:624-688, incorporated herein by reference. Using this

sequence, animal CDRs are distinguished from animal framework regions (FR) based on locations of the CDRs in known sequences of animal variable genes. Kabat, H.A., et al., Sequences of Proteins of Immunological Interest, 4th Ed. (U.S. Dept. Health and Human Services, Bethesda, MD, 1987). Once the animal CDRs and FR are identified, the CDRs are grafted onto human heavy chain variable region framework by the use of synthetic oligonucleotides and polymerase chain reaction (PCR) recombination. Codons for the animal heavy chain CDRs, as well as the available human heavy chain variable region framework, are built in four (each 100 bases long) oligonucleotides. Using PCR, a grafted DNA sequence of 400 bases is formed that encodes for the recombinant animal CDR/human heavy chain FR protection.

The immunogenic stimulus presented by the monoclonal antibodies so produced may be further decreased by the use of Pharmacia's (Pharmacia LKB Biotechnology, Sweden) "Recombinant Phage Antibody System" (RPAS), which generates a single-chain Fv fragment (ScFv) which incorporates the complete antigen-binding domain of the antibody. In the RPAS, antibody variable heavy and light chain genes are separately amplified from the hybridoma mRNA and cloned into an expression vector. The heavy and light chain domains are co-expressed on the same polypeptide chain after joining with a short linker DNA which codes for a flexible peptide. This assembly generates a single-chain Fv fragment (ScFv) which incorporates the complete antigen-binding domain of the antibody. Compared to the intact monoclonal antibody, the recombinant ScFv includes a considerably lower number of epitopes, and thereby presents a much weaker immunogenic stimulus when injected into humans.



The antibodies can be formulated in standard pharmaceutical carriers for administration to patients in need thereof. These include saline, phosphate buffered saline, and other aqueous carriers, and liposomes, polymeric microspheres and other controlled release delivery devices, as are well known in the art. The antibodies can also be administered with adjuvant, such as muramyl dipeptide or other materials approved for use in humans (Freund's adjuvant can be used for administration of antibody to animals).

Screening for drugs modifying or altering the extent of receptor function or expression

The receptor proteins are useful as targets for compounds which turn on, or off, or otherwise regulate binding to these receptors. The assays described above clearly provide routine methodology by which a compound can be tested for an inhibitory effect on binding of PC or APC. The *in vitro* studies of compounds which appear to inhibit binding selectively to the receptors are then confirmed by animal testing. Since the molecules are so highly evolutionarily conserved, it is possible to conduct studies in laboratory animals such as mice to predict the effects in humans.

In cases where inflammatory mediators or vascular disease down regulate EPCR, it would be advantageous to increase its concentration *in vivo* on endothelium. The binding assays described here and the gene sequence allow assays for increased EPCR expression. Similar approaches have been taken with thrombomodulin and investigators have shown that cyclic AMP (Maruyama, I. et al. (1991) Thrombosis Research 61, 301-310) and interleukin 4 (Kapiotis, S. et al., (1991) Blood 78, 410-415) can elevate thrombomodulin expression. The ability to

screen such drugs or drugs that block TNF down regulation of EPCR provide an approach to elevating EPCR expression *in vivo* and thus enhancing anticoagulant and anti-inflammatory activity.

5               Studies based on inhibition of binding are predictive for indirect effects of alteration of receptor binding. For example, inhibition of binding of APC or increased expression of TNF is predictive of inhibition of EPCR function.

10              Assays for testing compounds for useful activity can be based solely on interaction with the receptor protein, preferably expressed on the surface of transfected cells such as those described above. Proteins in solution or  
15              immobilized on inert substrates can also be utilized. These can be used to detect inhibition or enhancement in binding of PC or APC

                Alternatively, the assays can be based on interaction with the gene sequence encoding the  
20              receptor protein, preferably the regulatory sequences directing expression of the receptor protein. For example, antisense which binds to the regulatory sequences, and/or to the protein encoding sequences can be synthesized using  
25              standard oligonucleotide synthetic chemistry. The antisense can be stabilized for pharmaceutical use using standard methodology (encapsulation in a liposome or microsphere; introduction of modified nucleotides that are resistant to degradation or  
30              groups which increase resistance to endonucleases, such as phosphorothiodates and methylation), then screened initially for alteration of receptor activity in transfected or naturally occurring cells which express the receptor, then *in vivo* in  
35              laboratory animals. Typically, the antisense would inhibit expression. However, sequences which block

those sequences which "turn off" synthesis can also be targeted.

The receptor protein for study can be isolated from either naturally occurring cells or  
5 cells which have been genetically engineered to express the receptor, as described in the examples above. In the preferred embodiment, the cells would have been engineered using the intact gene.

10 *Random generation of receptor or receptor encoding sequence binding molecules.*

Molecules with a given function, catalytic or ligand-binding, can be selected for from a complex mixture of random molecules in what  
15 has been referred to as "in vitro genetics" (Szostak, (1992) TIBS 19:89). One synthesizes a large pool of molecules bearing random and defined sequences and subjects that complex mixture, for example, approximately  $10^{15}$  individual sequences in  
20 100  $\mu$ g of a 100 nucleotide RNA, to some selection and enrichment process. For example, by repeated cycles of affinity chromatography and PCR amplification of the molecules bound to the ligand on the column, Ellington and Szostak (1990)  
25 estimated that 1 in  $10^{10}$  RNA molecules folded in such a way as to bind a given ligand. DNA molecules with such ligand-binding behavior have been isolated (Ellington and Szostak, 1992; Bock et al, 1992).

30 *Computer assisted drug design*

Computer modeling technology allows visualization of the three-dimensional atomic structure of a selected molecule and the rational design of new compounds that will interact with the  
35 molecule. The three-dimensional construct typically depends on data from x-ray crystallographic analyses or NMR imaging of the selected molecule. The molecular dynamics require

force field data. The computer graphics systems enable prediction of how a new compound will link to the target molecule and allow experimental manipulation of the structures of the compound and target molecule to perfect binding specificity. Prediction of what the molecule-compound interaction will be when small changes are made in one or both requires molecular mechanics software and computationally intensive computers, usually coupled with user-friendly, menu-driven interfaces between the molecular design program and the user.

Examples of molecular modelling systems are the CHARMM and QUANTA programs, Polygen Corporation, Waltham, MA. CHARMM performs the energy minimization and molecular dynamics functions. QUANTA performs the construction, graphic modelling and analysis of molecular structure. QUANTA allows interactive construction, modification, visualization, and analysis of the behavior of molecules with each other.

A number of articles review computer modeling of drugs interactive with specific proteins, such as Rotivinen, et al., (1988) Acta Pharmaceutica Fennica 97, 159-166; Ripka, New Scientist 54-57 (June 16, 1988); McKinaly and Rossmann, (1989) Annu. Rev. Pharmacol. Toxicol. 29, 111-122; Perry and Davies, QSAR: Quantitative Structure-Activity Relationships in Drug Design pp. 189-193 (Alan R. Liss, Inc. 1989); Lewis and Dean, (1989) Proc. R. Soc. Lond. 236, 125-140 and 141-162; and, with respect to a model receptor for nucleic acid components, Askew, et al., (1989) J. Am. Chem. Soc. 111, 1082-1090. Other computer programs that screen and graphically depict chemicals are available from companies such as BioDesign, Inc., Pasadena, CA., Allelix, Inc., Mississauga, Ontario, Canada, and Hypercube, Inc.,

Cambridge, Ontario. Although these are primarily designed for application to drugs specific to particular proteins, they can be adapted to design of drugs specific to regions of DNA or RNA, once  
5 that region is identified.

Although described above with reference to design and generation of compounds which could alter binding, one could also screen libraries of known compounds, including natural products or  
10 synthetic chemicals, and biologically active materials, including proteins, for compounds which are inhibitors or activators.

*Generation of nucleic acid regulators*

Nucleic acid molecules containing the 5'  
15 regulatory sequences of the receptor genes can be used to regulate or inhibit gene expression in vivo. Vectors, including both plasmid and eukaryotic viral vectors, may be used to express a particular recombinant 5' flanking region-gene  
20 construct in cells depending on the preference and judgment of the skilled practitioner (see, e.g., Sambrook et al., Chapter 16). Furthermore, a number of viral and nonviral vectors are being developed that enable the introduction of nucleic  
25 acid sequences in vivo (see, e.g., Mulligan, (1993) Science, 260, 926-932; United States Patent No. 4,980,286; United States Patent No. 4,868,116; incorporated herein by reference). Recently, a delivery system was developed in which nucleic acid  
30 is encapsulated in cationic liposomes which can be injected intravenously into a mammal. This system has been used to introduce DNA into the cells of multiple tissues of adult mice, including endothelium and bone marrow (see, e.g., Zhu et al.,  
35 (1993) Science 261, 209-211; incorporated herein by reference).

The 5' flanking sequences of the receptor gene can also be used to inhibit the expression of the receptor. For example, an antisense RNA of all or a portion of the 5' flanking region of the receptor gene can be used to inhibit expression of the receptor *in vivo*. Expression vectors (e.g., retroviral expression vectors) are already available in the art which can be used to generate an antisense RNA of a selected DNA sequence which is expressed in a cell (see, e.g., U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286). Accordingly, DNA containing all or a portion of the sequence of the 5' flanking region of the receptor gene can be inserted into an appropriate expression vector so that upon passage into the cell, the transcription of the inserted DNA yields an antisense RNA that is complementary to the mRNA transcript of the receptor protein gene normally found in the cell. This antisense RNA transcript of the inserted DNA can then base-pair with the normal mRNA transcript found in the cell and thereby prevent the mRNA from being translated. It is of course necessary to select sequences of the 5' flanking region that are downstream from the transcriptional start sites for the receptor protein gene to ensure that the antisense RNA contains complementary sequences present on the mRNA.

Antisense RNA can be generated *in vitro* also, and then inserted into cells. Oligonucleotides can be synthesized on an automated synthesizer (e.g., Model 8700 automated synthesizer of Milligen-Bioscience, Burlington, MA or ABI Model 380B). In addition, antisense deoxyoligonucleotides have been shown to be effective in inhibiting gene transcription and viral replication (see e.g., Zamecnik et al.,

- (1978) Proc. Natl. Acad. Sci. USA 75, 280-284; Zamecnik et al., (1986) Proc. Natl. Acad. Sci., 83, 4143-4146; Wickstrom et al., (1988) Proc. Natl. Acad. Sci. USA 85, 1028-1032; Crooke, (1993) FASEB J. 7, 533-539. Furthermore, recent work has shown that improved inhibition of expression of a gene by antisense oligonucleotides is possible if the antisense oligonucleotides contain modified nucleotides (see, e.g., Offensperger et. al., (1993) EMBO J. 12, 1257-1262 (in vivo inhibition of duck hepatitis B viral replication and gene expression by antisense phosphorothioate oligodeoxynucleotides); PCT WO 93/01286 Rosenberg et al., (synthesis of sulfurthioate oligonucleotides); Agrawal et al., (1988) Proc. Natl. Acad. Sci. USA 85, 7079-7083 (synthesis of antisense oligonucleoside phosphoramidates and phosphorothioates to inhibit replication of human immunodeficiency virus-1); Sarin et al., (1989) Proc. Natl. Acad. Sci. USA 85, 7448-7794 (synthesis of antisense methylphosphonate oligonucleotides); Shaw et al., (1991) Nucleic Acids Res 19, 747-750 (synthesis of 3' exonuclease-resistant oligonucleotides containing 3' terminal phosphoroamidate modifications); incorporated herein by reference).

The sequences of the 5' flanking region of receptor protein gene can also be used in triple helix (triplex) gene therapy. Oligonucleotides complementary to gene promoter sequences on one of the strands of the DNA have been shown to bind promoter and regulatory sequences to form local triple nucleic acid helices which block transcription of the gene (see, e.g., Maher et al., (1989) Science 245, 725-730; Orson et al., (1991) Nucl. Acids Res. 19, 3435-3441; Postal et al., (1991) Proc. Natl. Acad. Sci. USA 88, 8227-8231;

- Cooney et al., (1988) Science 241, 456-459; Young et al., (1991) Proc. Natl. Acad. Sci. USA 88, 10023-10026; Duval-Valentin et al., (1992) Proc. Natl. Acad. Sci. USA 89, 504-508; Blume et al.,  
5 (1992) Nucl. Acids Res. 20, 1777-1784; Grigoriev et al., (1992) J. Biol. Chem. 267, 3389-3395.

Recently, both theoretical calculations and empirical findings have been reported which provide guidance for the design of oligonucleotides  
10 for use in oligonucleotide-directed triple helix formation to inhibit gene expression. For example, oligonucleotides should generally be greater than 14 nucleotides in length to ensure target sequence specificity (see, e.g., Maher et al., (1989);  
15 Grigoriev et al., (1992)). Also, many cells avidly take up oligonucleotides that are less than 50 nucleotides in length (see e.g., Orson et al., (1991); Holt et al., (1988) Mol. Cell. Biol. 8, 963-973; Wickstrom et al., (1988) Proc. Natl. Acad. Sci. USA 85, 1028-1032). To reduce susceptibility  
20 to intracellular degradation, for example by 3' exonucleases, a free amine can be introduced to a 3' terminal hydroxyl group of oligonucleotides without loss of sequence binding specificity (Orson et al., 1991). Furthermore, more stable triplexes  
25 are formed if any cytosines that may be present in the oligonucleotide are methylated, and also if an intercalating agent, such as an acridine derivative, is covalently attached to a 5' terminal  
30 phosphate (e.g., via a pentamethylene bridge); again without loss of sequence specificity (Maher et al., (1989); Grigoriev et al., (1992)).

Methods to produce or synthesize oligonucleotides are well known in the art. Such  
35 methods can range from standard enzymatic digestion followed by nucleotide fragment isolation (see e.g., Sambrook et al., Chapters 5, 6) to purely



synthetic methods, for example, by the cyanoethyl phosphoramidite method using a Milligen or Beckman System 1Plus DNA synthesizer (see also, Ikuta et al., (1984) Ann. Rev. Biochem. 53, 323-356  
5 (phosphotriester and phosphite-triester methods); Narang et al., (1980) Methods Enzymol., 65, 610-620 (phosphotriester method). Accordingly, DNA sequences of the 5' flanking region of the receptor protein gene described herein can be used to design  
10 and construct oligonucleotides including a DNA sequence consisting essentially of at least 15 consecutive nucleotides, with or without base modifications or intercalating agent derivatives, for use in forming triple helices specifically  
15 within the 5' flanking region of a receptor protein gene in order to inhibit expression of the gene.

In some cases it may be advantageous to insert enhancers or multiple copies of the regulatory sequences into an expression system to  
20 facilitate screening of methods and reagents for manipulation of expression.

#### *Preparation of Receptor Protein Fragments*

Compounds which are effective for blocking binding of the receptor can also consist  
25 of fragments of the receptor proteins, expressed recombinantly and cleaved by enzymatic digest or expressed from a sequence encoding a peptide of less than the full length receptor protein. These will typically be soluble proteins, i.e., not  
30 including the transmembrane and cytoplasmic regions, although smaller portions determined in the assays described above to inhibit or compete for binding to the receptor proteins can also be utilized. It is a routine matter to make  
35 appropriate receptor protein fragments, test for binding, and then utilize. The preferred fragments are of human origin, in order to minimize potential

immunological response. The peptides can be as short as five to eight amino acids in length and are easily prepared by standard techniques. They can also be modified to increase *in vivo* half-life, by chemical modification of the amino acids or by attachment to a carrier molecule or inert substrate. Based on studies with other peptide fragments blocking receptor binding, the  $IC_{50}$ , the dose of peptide required to inhibit binding by 50%, ranges from about 1  $\mu$ M to greater than 10 mM, depending on the peptide size and folding. These ranges are well within the effective concentrations for the *in vivo* administration of peptides, based on comparison with the RGD-containing peptides, described, for example, in U.S. Patent No. 4,792,525 to Ruoslahti, et al., used *in vivo* to alter cell attachment and phagocytosis. The peptides can also be conjugated to a carrier protein such as keyhole limpet hemocyanin by its N-terminal cysteine by standard procedures such as the commercial Imject kit from Pierce Chemicals or expressed as a fusion protein, which may have increased efficacy.

As noted above, the peptides can be prepared by proteolytic cleavage of the receptor proteins, or, preferably, by synthetic means. These methods are known to those skilled in the art. An example is the solid phase synthesis described by J. Merrifield, (1964) J. Am. Chem. Soc. 85, 2149, used in U.S. Patent No. 4,792,525, and described in U.S. Patent No. 4,244,946, wherein a protected alpha-amino acid is coupled to a suitable resin, to initiate synthesis of a peptide starting from the C-terminus of the peptide. Other methods of synthesis are described in U.S. Patent No. 4,305,872 and 4,316,891. These methods can be used to synthesize peptides having identical

sequence to the receptor proteins described herein, or substitutions or additions of amino acids, which can be screened for activity as described above.

The peptide can also be administered as a  
5 pharmaceutically acceptable acid- or base- addition salt, formed by reaction with inorganic acids such as hydrochloric acid, hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, and phosphoric acid, and organic acids such as  
10 formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic acid, maleic acid, and fumaric acid, or by reaction with an inorganic base such as sodium hydroxide, ammonium hydroxide,  
15 potassium hydroxide, and organic bases such as mono-, di-, trialkyl and aryl amines and substituted ethanolamines.

Peptides containing cyclopropyl amino acids, or amino acids derivatized in a similar  
20 fashion, can also be used. These peptides retain their original activity but have increased half-lives *in vivo*. Methods known for modifying amino acids, and their use, are known to those skilled in the art, for example, as described in U.S. Patent  
25 No. 4,629,784 to Stammer.

The peptides are generally active when administered parenterally in amounts above about 1  $\mu\text{g/kg}$  of body weight. Based on extrapolation from other proteins, for treatment of most inflammatory  
30 disorders, the dosage range will be between 0.1 to 70  $\text{mg/kg}$  of body weight. This dosage will be dependent, in part, on whether one or more peptides are administered.

#### *Pharmaceutical Compositions*

35 Compounds which alter receptor protein binding are preferably administered in a pharmaceutically acceptable vehicle. Suitable

pharmaceutical vehicles are known to those skilled in the art. For parenteral administration, the compound will usually be dissolved or suspended in sterile water or saline. For enteral

- 5 administration, the compound will be incorporated into an inert carrier in tablet, liquid, or capsular form. Suitable carriers may be starches or sugars and include lubricants, flavorings, binders, and other materials of the same nature.
- 10 The compounds can also be administered locally by topical application of a solution, cream, gel, or polymeric material (for example, a Pluronic™, BASF).

- Alternatively, the compound may be
- 15 administered in liposomes or microspheres (or microparticles). Methods for preparing liposomes and microspheres for administration to a patient are known to those skilled in the art. U.S. Patent No. 4,789,734 describe methods for encapsulating
- 20 biological materials in liposomes. Essentially, the material is dissolved in an aqueous solution, the appropriate phospholipids and lipids added, along with surfactants if required, and the material dialyzed or sonicated, as necessary. A
- 25 review of known methods is by G. Gregoriadis, Chapter 14. "Liposomes", Drug Carriers in Biology and Medicine pp. 287-341 (Academic Press, 1979). Microspheres formed of polymers or proteins are well known to those skilled in the art, and can be
- 30 tailored for passage through the gastrointestinal tract directly into the bloodstream.
- Alternatively, the compound can be incorporated and the microspheres, or composite of microspheres, implanted for slow release over a period of time,
- 35 ranging from days to months. See, for example, U.S. Patent Nos. 4,906,474, 4,925,673, and 3,625,214.

*Disorders to be treated*

As described herein, a variety of compounds can be used to inhibit or enhance expression of the EPCR. The nature of the disorder will determine if the expression should be enhanced or inhibited. For example, based on the studies involving the use of an anti-protein C antibody in combination with cytokine, it should be possible to treat solid tumors by enhancing an inflammatory response involving blocking of protein C or activated protein C binding to an endothelial cell protein C/activated protein C receptor by administering to a patient in need of treatment thereof an amount of a compound blocking binding of protein C or activated protein C to the receptor. Similarly, it should be possible to treat disorders such as gram negative sepsis, stroke, thrombosis, septic shock, adult respiratory distress syndrome, and pulmonary emboli using a method for inhibiting an inflammatory response involving administration of EPCR or EPCR fragments or substances that upregulate EPCR expression to a patient in need of treatment thereof.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Oklahoma Medical Research Foundation  
(ii) TITLE OF INVENTION: Cloning and Regulation of an Endothelial Cell Protein C/Activated Protein C Receptor  
(iii) NUMBER OF SEQUENCES: 2  
(iv) CORRESPONDENCE ADDRESS:

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(C) CITY: Atlanta  
(D) STATE: Georgia  
(E) COUNTRY: USA  
(F) ZIP: 30309-3450

## (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

## (viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Pabst, Patrea L.  
(B) REGISTRATION NUMBER: 31,284  
(C) REFERENCE/DOCKET NUMBER: OMRF152

## (ix) TELECOMMUNICATION INFORMATION:

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## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1302 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

## (ix) FEATURE:

(A) NAME/KEY: misc\_feature

(B) LOCATION: 1..1302

(D) OTHER INFORMATION: /note= "Nucleotides 25 through 738 encode the Endothelial Cell Protein Receptor of Sequence ID No. 2."  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CAGGTCCGGA	GCCTCAACTT	CAGGATGTTG	ACAACATTGC	TGCCGATACT	GCTGCTGTCT	60
GGCTGGGCCT	TTTGTAGCCA	AGACGCCCTCA	GATGGCCTCC	AAAGACTTCA	TATGCTCCAG	120
ATCTCCTACT	TCCGGGACCC	CTATCACGTG	TGGTACCAGG	GCAACGCGTC	GCTGGGGGGA	180
CACCTAACGC	ACGTGCTGGA	AGGCCCAGAC	ACCAACACCA	CGATCATTTCA	GCTGCAGCCC	240
TTGCAGGAGC	CCGAGAGCTG	GGCGGCACG	CAGAGTGGCC	TGCAGTCCTA	CCTGCTCCAG	300
TTCCACGGCC	TCGTGGGCCT	GGTGCACCAG	GAGCGGACCT	TGGCCTTTCC	TCTGACCATC	360
CGCTGCTTCC	TGGGCTGTGA	GCTGCCTCCC	GAGGGCTCTA	GAGCCCATGT	CTTCTTCGAA	420
GTGGCTGTGA	ATGGGAGCTC	CTTTGTGAGT	TTCCGGGCCGG	AGAGAGCCTT	GTGGCAGGCA	480
GACACCCAGG	TCACCTCCGG	AGTGGTCACC	TTCACCCCTGC	AGCAGCTCAA	TGCCTACAAC	540
CGCACTCGGT	ATGAACTGCG	GGAATTCCTG	GAGGACACCT	GTGTGCAGTA	TGTGCAGAAA	600
CATATTTCCG	CGGAAAACAC	GAAAGGGAGC	CAAACAAGCC	GCTCCTACAC	TTGCTGTGTC	660
CTGGGCGTCC	TGGTGGGCGG	TTTCATCATT	GCTGGTGTGG	CTGTAGGCAT	CTTCCTGTGC	720
ACAGGTGGAC	GGCGATGTTA	ATTACTCTCC	AGCCCCGTCA	GAAAGGGGCTG	GATTGATGGA	780
GGCTGGCAAG	GGAAAGTTTC	AGCTCACTGT	GAAGCCAGAC	TCCCCAACTG	AAACACCAGA	840
AGGTTTGGAG	TGACAGCTCC	TTTCTTCTCC	CACATCTGCC	CACTGAAGAT	TTGAGGGGAGG	900
GGAGATGGAG	AGGAGAGGTG	GACAAAGTAC	TTGGTTTGCT	AAGAACCTAA	GAACGTGTAT	960

GCTTTGCTGA	ATTAGTCTGA	TAAGTGAATG	TTTATCTATC	TTTGTGGAAA	ACAGATAATG	1020
GAGTTGGGGC	AGGAAGCCCTA	TGCGCCATCC	TCCAAAGACA	GACAGAATCA	CCTGAGGCGT	1080
TCAAAAGATA	TAACCAAATA	AACAAGTCAT	CCACAATCAA	AATACAACAT	TCAATACTTC	1140
CAGGTGTGTC	AGACTTGGGA	TGGGACGCTG	ATATAATAGG	GTAGAAAGAA	GTAACACGAA	1200
GAAGTGGTGG	AAATGTAAAA	TCCAAGTCAT	ATGGCAGTGA	TCAATTATTA	ATCAATTAAAT	1260
AATATTAAATA	AATTTCTTAT	ATTAAAAAAA	AAAAAAAAAA	AA		1302

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 238 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(ix) FEATURE:

(A) NAME/KEY: misc feature

(B) LOCATION: 1..365

(D) OTHER INFORMATION: /note= "Endothelial Cell Protein Receptor encoded by nucleotides 1 through 1302 of Sequence ID No. 1."

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 1..15

(D) OTHER INFORMATION: /note= "Amino acids 1-15 represent a putative signal sequence."

(ix) FEATURE:

(A) NAME/KEY: Domain

(B) LOCATION: 211..236

(D) OTHER INFORMATION: /note= "Amino acids 211-236 represent a putative transmembrane domain."

(ix) FEATURE:

(A) NAME/KEY: Active-site

(B) LOCATION: 47..174



(D) OTHER INFORMATION: /note= "Amino acids 47-49, 64-66, 136-138 and 172-174 represent potential N-glycosylation sites."

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 17..186

(D) OTHER INFORMATION: /note= "Amino acids 17, 114, 118 and 186 represent extracellular cysteine residues."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```

Met Leu Thr Thr Leu Leu Pro Ile Leu Leu Leu Ser Gly Trp Ala Phe
 1      5      10      15

Cys Ser Gln Asp Ala Ser Asp Gly Leu Gln Arg Leu His Met Leu Gln
20      25      30

Ile Ser Tyr Phe Arg Asp Pro Tyr His Val Trp Tyr Gln Gly Asn Ala
35      40      45

Ser Leu Gly Gly His Leu Thr His Val Leu Glu Gly Pro Asp Thr Asn
50      55      60

Thr Thr Ile Ile Gln Leu Leu Gln Pro Leu Gln Glu Pro Glu Ser Trp Ala
65      70      75      80

Arg Thr Gln Ser Gly Leu Leu Gln Ser Tyr Leu Leu Gln Phe His Gly Leu
85      90      95

Val Arg Leu Val His Gln Glu Arg Thr Leu Ala Phe Pro Leu Thr Ile
100      105      110

Arg Cys Phe Leu Gly Cys Glu Leu Pro Pro Glu Gly Ser Arg Ala His
115      120      125

Val Phe Phe Glu Val Ala Val Asn Gly Ser Ser Phe Val Ser Phe Arg
130      135      140

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Pro Glu Arg Ala Leu Trp Gln Ala Asp Thr Gln Val Thr Ser Gly Val  
 145 150 155 160  
 Val Thr Phe Thr Leu Gln Gln Leu Asn Ala Tyr Asn Arg Thr Arg Tyr  
 165 170 175  
 Glu Leu Arg Glu Phe Leu Glu Asp Thr Cys Val Gln Tyr Val Gln Lys  
 180 185 190  
 His Ile Ser Ala Glu Asn Thr Lys Gly Ser Gln Thr Ser Arg Ser Tyr  
 195 200 205  
 Thr Ser Leu Val Leu Gly Val Leu Val Gly Gly Phe Ile Ile Ala Gly  
 210 215 220  
 Val Ala Val Gly Ile Phe Leu Cys Thr Gly Gly Arg Arg Cys  
 225 230 235

We claim.

1. An isolated endothelial cell protein C/activated protein C receptor.

2. The receptor of claim 1 encoded by the nucleotide sequence of Sequence ID No. 1 and degenerative sequences thereof and sequence having conservative substitutions, additions or deletions thereof hybridizing to Sequence ID No. 1 under stringent conditions, which encode the receptor.

3. The receptor of claim 1 having the amino acid sequence of Sequence ID No. 2 or a sequence having conservative substitutions, additions or deletions thereof.

4. The receptor of claim 1 expressed on the surface of a non-human cell or a non-endothelial cell.

5. The receptor of claim 1 in soluble form.

6. The receptor of claim 5 lacking at least a portion of the transmembrane region.

7. A nucleotide sequence encoding an endothelial cell protein C/activated protein C receptor.

8. The sequence of claim 7 having the nucleotide sequence of Sequence ID No. 1 or degenerative sequences thereof and sequence having conservative substitutions, additions or deletions thereof and hybridizing under stringent conditions to Sequence ID No. 1.

9. The sequence of claim 7 encoding the amino acid sequence of Sequence ID No. 2 or a sequence having conservative substitutions, additions or deletions thereof.

10. The sequence of claim 7 further comprising an expression vector.

11. The sequence of claim 10 further comprising an expression host.

12. The sequence of claim 10 expressed on the surface of a non-human cell or a non-endothelial cell.

13. The sequence of claim 7 encoding a soluble form of the receptor.

14. The sequence of claim 7 encoding a fragment of the receptor of at least fourteen consecutive nucleotides in length.

15. The sequence of claim 14 labelled with a detectable label.

16. A method for enhancing an inflammatory response involving blocking of protein C or activated protein C binding to an endothelial cell protein C/activated protein C receptor comprising administering to a patient in need of treatment thereof an amount of a compound blocking binding of protein C or activated protein C to the receptor. tumors

17. The method of claim 16 wherein the compound is selected from the group consisting of antibodies and fragments of antibodies to the receptor, nucleic acid sequences inhibiting expression of the receptor, and synthetic or natural compounds other than proteins, peptides or nucleic acid sequences which inhibit binding.

18. A method for inhibiting an inflammatory response involving administration of a compound selected from the group consisting of EPCR or EPCR fragments and substances that upregulate EPCR expression to a patient in need of treatment thereof.

19. An antibody or antibody fragment specifically immunoreactive with a unique epitope of an isolated endothelial cell protein C/activated prot in C receptor.

20. The antibody of claim 19 wherein the receptor is encoded by the nucleotide sequence of

Sequence ID No. 1 and degenerative sequences thereof and sequence having conservative substitutions, additions or deletions thereof and hybridizing to Sequence ID No. 1 under stringent conditions.

21. The antibody of claim 19 wherein the receptor has the amino acid sequence of Sequence ID No. 2 or a sequence having conservative substitutions, additions or deletions thereof.

22. A method for screening for a compound which alters the binding of an endothelial receptor protein to protein C or activated protein C comprising providing an assay for binding of protein C or activated protein C to the receptor protein,

adding the compound to be tested to the assay, and

determining if the amount of protein C or activated protein C which is bound to the receptor protein is altered as compared to binding in the absence of the compound to be tested.

23. A method for screening patients for abnormal receptor protein activity or function comprising

determining the presence of an endothelial cell surface receptor binding protein C and activated protein C, and

comparing the receptor to determine if the quantity present or the function of the receptor is equivalent to that present in normal cells.

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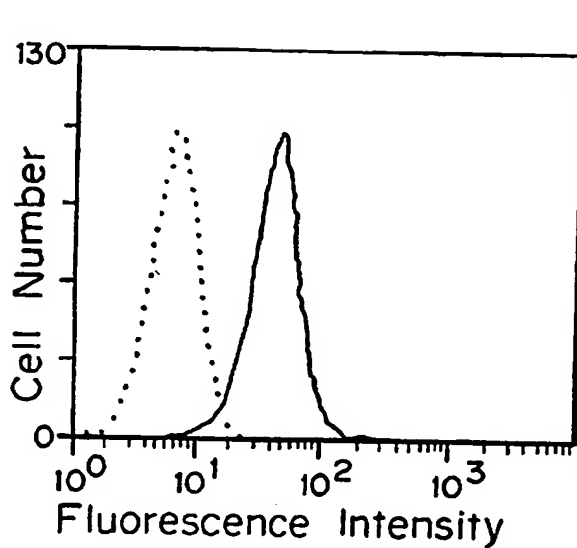


FIG. 1a

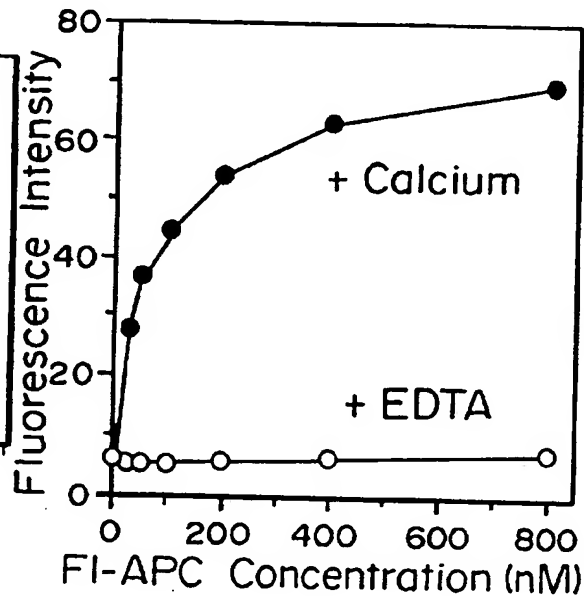


FIG. 1b

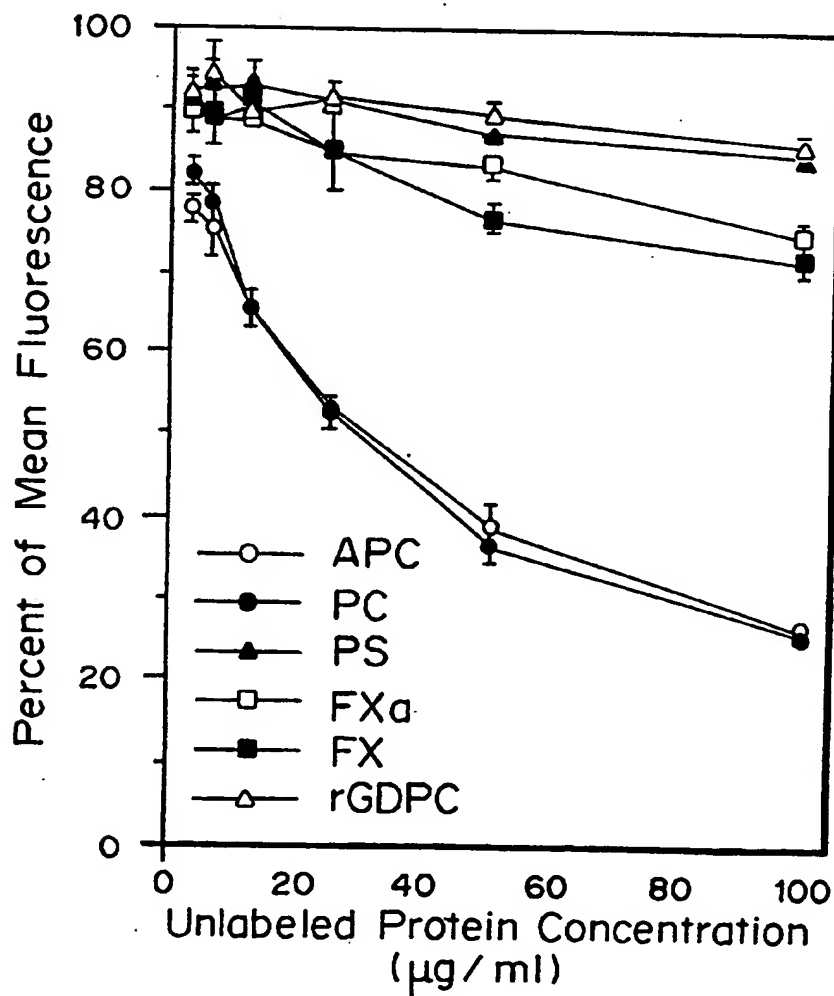


FIG. 1c

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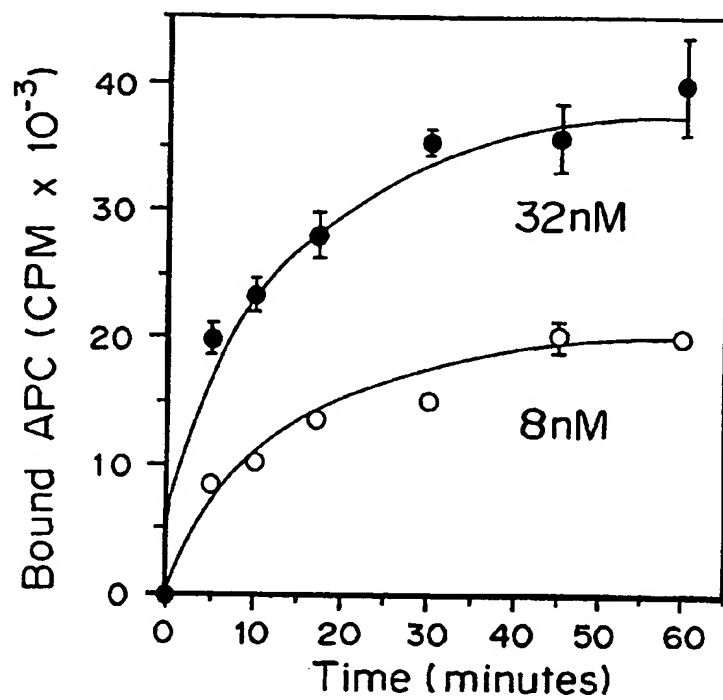


FIG. 2a

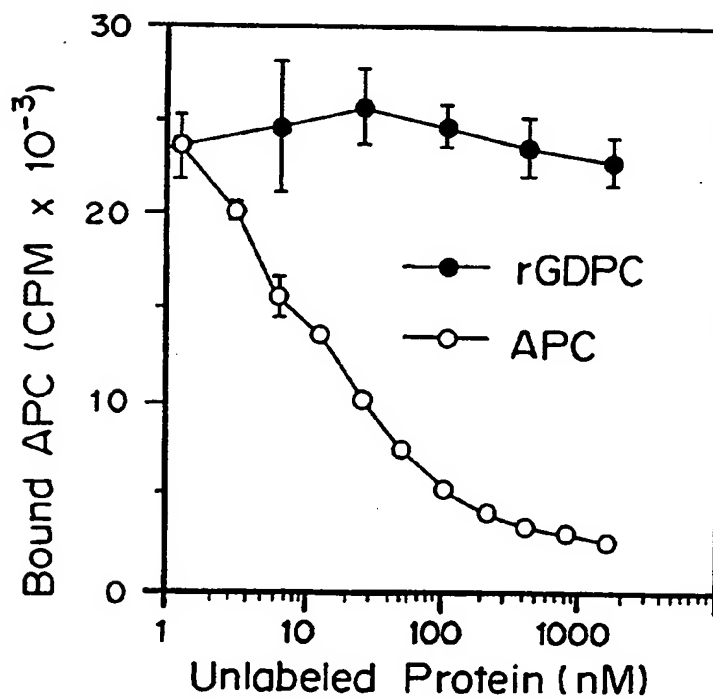


FIG. 2b

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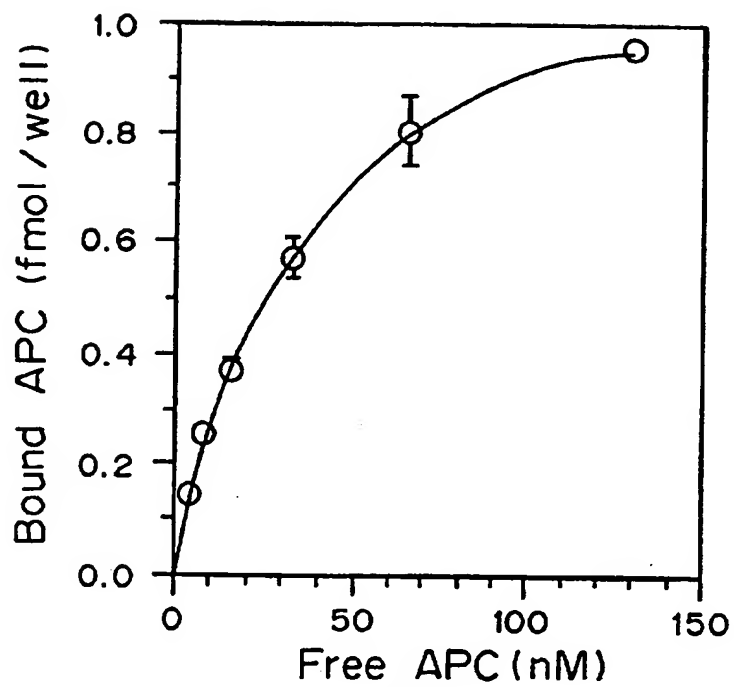


FIG. 2c

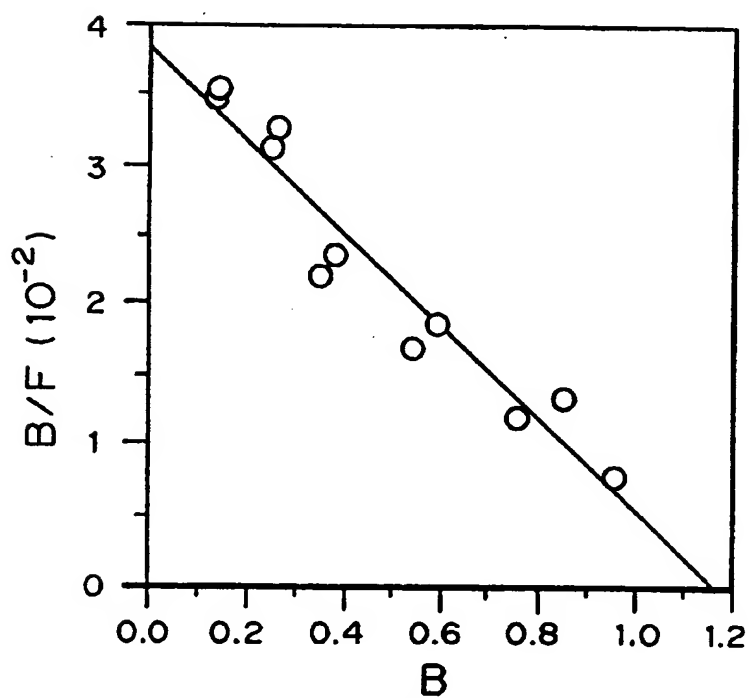


FIG. 2d



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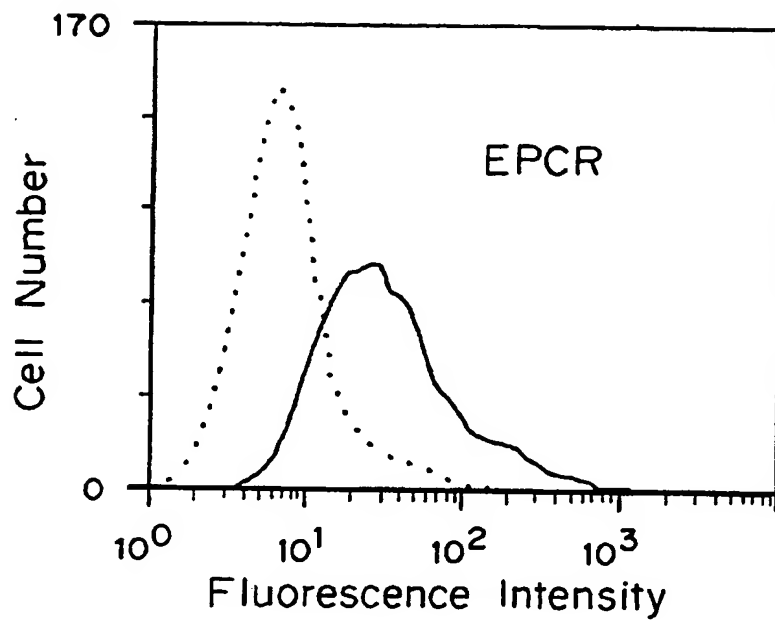


FIG. 3a

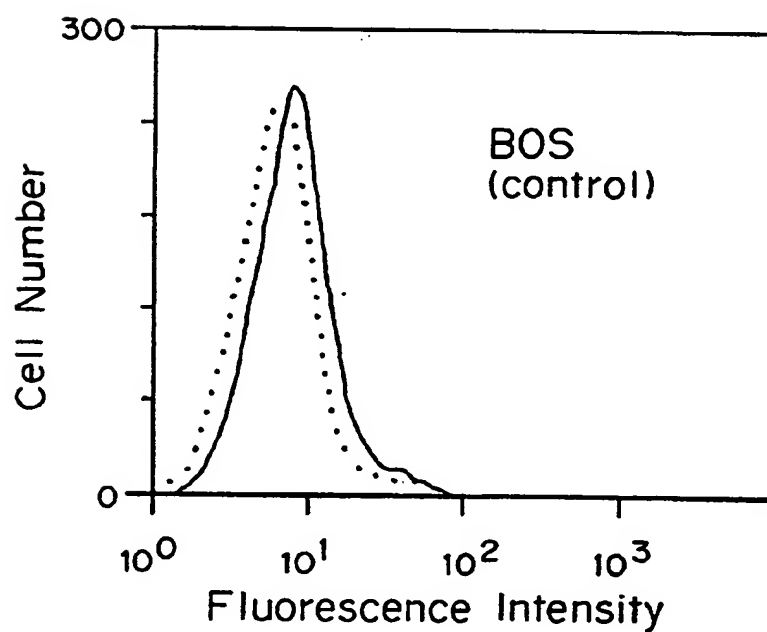


FIG. 3b

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FIG. 4a

CAGGTCCGGAGCCTCAACTTCAGGATGTTGACAACATTGCTGCCGATACTGCTGCTGTCT 60  
M L T T L L P I L L L S 12

GGCTGGGCGCTTTGTAGCCAAGACGGCTCAGATGGCCCTCCAAAGACTTCATATGCTCCAG 120  
G W A F ③ S Q D A S D G L Q R L H M L Q 32

ATCTCCTACTTCGGGACCCCTATCAGGTGTGGTACCAGGGCAACGG TCGCTGGGGGA 180  
 I S Y F R D P Y H V W Y Q G N A S L G G 52

CAGCTAAGCAGGTGCTGGAAGGCCGAGACCAACACGACGATCATTAGCTGCAGCCG 240  
 H L T H V L E G P D T N T T I I Q L Q P 72

TTGCAGGAGCCGAGAGCTGGGGCGGACGAGAGTGGCCTGCAGTCTAGCTGCTCCAG 300  
 L Q E P E S W A R T Q S G L Q S Y L L Q 92

TTCCACGGCCTGCTGGCCTGGTGCCAGCAGGAGGGACCTTGGCCTTCCCTCTGACCATC 360  
 F H G L V R L V H Q E R T L A F P L T I 112

CGCTGCTTCCCTGGGCTGTAGCTGCCCTCCGAGGGCTCTAGAGCCCATGTCTTCTCGAA 420  
 R ③ F L G ③ E L P P E G S R A H V F F E 132

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FIG. 4b

GTGGCTGTAATGGAGCTCCTTTGTGAGTTTCGGCGGAGAGCCTTGTGGCAGGCA 480  
 V A V N G S S F V S F R P E R A L W Q A 152

GACACCCAGGTCACCTCGGGAGTGGTCACCTTCACCCCTGCAGCAGCTCAATGCCTACAAC 540  
 D T Q V T S G V V T F T L Q Q L N A Y N 172

CGCACTCGGTATGAAC TCGGGGAATTCCTGGAGGACACCTGTGTGCAGTATGTGCAGAAA 600  
R T R Y E L R E F L E D T C V Q Y V Q K 192

CATATTCGGCGGAAACACGAAAGGGAGGCCAAACAAGCGCTCCTACACTTCGCTGGTC 660  
 H I S A E N T K G S Q T S R S Y T S L V 212

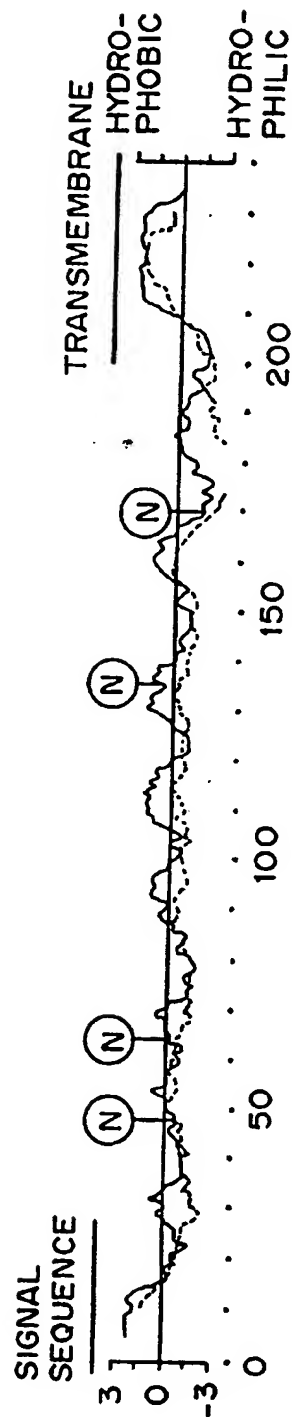
CTGGGCGTCCTGGCGGGTTTCATCATTGCTGGTGTGGCTGTAGGCATCTTCCTGTGC 720  
 L G V L V G G F I I A G V A V G I F L C 232

ACAGGTGACGGGATGTTAATTACTCTCCAGCCCGCTCAGAAGGGCTGGATTGATGGA 780  
T G G R R C \* 238

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FIG. 4c

GGCTGGCAAGGGAAGTTTCAGCTCACTGTGAAGCCAGACTCCCCAACTGAAACACCAGA 840  
AGGTTTGGAGTGACAGCTCCTTCTCTCCACATCTGCCCACTGAAGATTGAGGGAGG 900  
GGAGATGGAGAGGAGAGGTGGACAAAGTACTTGGTTTGCTAAGAACCTAAGAACGTGTAT 960  
GCTTTGCTGAATTAGTCTGATAAGTGAATGTTTATCTATCTTTGTGGAAACAGATAATG 1020  
GAGTTGGGCGCAGGAGCCTATGCGGCATCCTCCAAAGACAGACAGAAATCACCTGAGGCGT 1080  
TCAAAGATATAACCAAATAACAAGTCATCCACAATCAAAATACAACATTCAATCTTC 1140  
CAGGTGTGCAGACTTGGGATGGGAGCGTGATATAATAGGGTAGAAAGTAACACGAA 1200  
GAAGTGGTGGAAATGTAAATCCAAAGTCATATGGCAGTGATCAATTATTATCAATTAAT 1260  
AATATTAATAAATTTCTATATTTAAAAAATAAAAAA 1302





EPCR  
 CCD41  
 CD1d  
 MCD1.2

**SUBSTITUTE SHEET (RULE 26)**



# INTERNATIONAL SEARCH REPORT

Internat'l Application No

PCT/US 95/09636

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C07K14/705 A61K39/395 C12N15/11 A61K38/17  
C07K16/28 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 42, 21 October 1994 BALTIMORE, MD, USA, pages 26486-26491, K. FUKUDOME ET AL. 'Identification, cloning, and regulation of a novel endothelial cell protein C/activated protein C receptor.' see the whole document --- -/--</p>	1-3,7-11

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

### \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- \*Z\* document member of the same patent family

Date of the actual completion of the international search

2 January 1996

Date of mailing of the international search report

26.01.96

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,  
Fax (+ 31-70) 340-3016

Authorized officer

Nooij, F



## INTERNATIONAL SEARCH REPORT

Internat'l Application No

PCT/US 95/09636

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>CIRCULATION, v 1. 90, no. 4 part 2, October 1994 NEW YORK, NY, USA, page I-N K. FUKUDOME ET AL. 'Identification, cloning, and regulation of a novel endothelial cell protein C/activated protein C receptor.' see the whole document ---</p>	1-3,7-11
P,X	<p>CIRCULATION, vol. 90, no. 4 part 2, October 1994 NEW YORK, NY, USA, page I133 K. FUKUDOME ET AL. 'Identification, cloning and regulation of a novel endothelial cell protein C/activated protein C receptor.' see abstract 0707 ---</p>	1-3,7-11
P,X	<p>THROMBOSIS AND HAEMOSTASIS, vol. 72, no. 3, September 1994 STUTTGART, GERMANY, pages 465-474, N. BANGALORE ET AL. 'High affinity binding sites for activated protein C and protein C on cultured human umbilical vein endothelial cells.' see summary see page 472, right column, line 9 - line 50 ---</p>	1-3,7-11
P,X	<p>THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 10, 10 March 1995 BALTIMORE, MD, USA, pages 5571-5577, K. FUKUDOME ET AL. 'Molecular cloning and expression of murine and bovine endothelial cell protein C/activated protein C receptor (EPCR).' see abstract see figures 1-5 -----</p>	1-4,7-12

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 95/09636

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 16-18  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although these claims are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.